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Intestinal Secretagogues Increase Cytosolic Free Ca^{2+} Concentration and K^+ Conductance in a Human Intestinal Epithelial Cell Line

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Summary. A human intestinal epithelial cell line (Intestine 407) is known to retain receptors for intestinal secretagogues such as acetylcholine (ACh), histamine, serotonin (5-HT) and vasoactive intestinal peptide (VIP). The cells were also found to possess separate receptors for secretin and ATP, the stimulation of which elicited transient hyperpolarizations coupled to decreased membrane resistances. These responses were reversed in polarity at the K^+ equilibrium potential. The hyperpolarizing responses to six agonists were reversibly inhibited by quinine or quinidine. By means of Ca^{2+}-selective microelectrodes, increases in the cytosolic free Ca^{2+} concentration were observed in response to individual secretagogues. The time course of Ca^{2+} responses coincided with that of hyperpolarizing responses. The responses to ACh and 5-HT were abolished by a reduction in the extracellular Ca^{2+} concentration down to pCa 7 or by application of Co^{2+}. Thus, in Intestine 407 cells, not only the intestinal secretagogues, which are believed to act via increased cytosolic Ca^{2+} (ACh, 5-HT and histamine), but also those which elevate cyclic AMP (VIP, secretin and ATP) induce increases in cytosolic Ca^{2+}, thereby activating the K^+ conductance. It is likely that the origin of increased cytosolic Ca^{2+} is mainly extracellular for ACh- and 5-HT-induced responses, whereas histamine, VIP, secretin and ATP mobilize Ca^{2+} from the internal compartment.

Key Words intestinal secretagogues · receptor · cytosolic Ca · K channel · intestinal epithelial cell

Introduction

Mechanisms underlying small intestinal secretion have extensively been investigated at the tissue level *in vivo* and *in vitro*. However, the cellular mechanisms were not fully elucidated, because of the structural and functional heterogeneity of the epithelium where multiple types of cells take part in opposite transport functions, absorption and secretion. To overcome this difficulty, we employed an epithelial cell line, Intestine 407, established from human embryonic small intestine (Henle & Deinhardt, 1957), to conduct electrophysiological studies on ionic mechanisms of the stimulation-secretion coupling at the cellular level. This cell line was selected as a model for Cl^--secreting epithelial cells of the small intestine on the following grounds: (i) This cell line is clearly distinct from a prevailing epithelial cell line, HeLa, with respect to electrical and enzymatic activities of the cell membrane (Yada & Okada, 1984; Hazama, Yada & Okada, 1985; Okada, Hazama & Yada, 1985); (ii) Intestine 407 cells retain receptors for a variety of intestinal secretagogues, including acetylcholine (ACh), serotonin (5-HT), histamine and vasoactive intestinal peptide (VIP) (Yada & Okada, 1984); (iii) In addition, this cell line has the binding site to heat-stable enterotoxin of *Escherichia coli* (Thomas & Knoop, 1983), another intestinal secretagogue whose action is mediated by cyclic GMP (Hughes et al., 1978); (iv) Cl^- currents can be induced in Intestine 407 cells by intestinal secretagogues under appropriate conditions (Itoh, Ueda & Okada, 1989).

Intestine 407 cells are known to respond with slow hyperpolarizations to ACh, 5HT and histamine by increasing the membrane K^+ conductance (Yada & Okada, 1984). Since the secretagogue action of ACh, 5HT and histamine is known to be Ca^{2+}-mediated (for review *see* Donowitz & Welsh, 1987), Ca^{2+}-activated K^+ channels appear to be responsible for the hyperpolarizing responses. In fact, similar hyperpolarizations were induced by the application of a Ca^{2+} ionophore A23187 (Yada & Okada, 1984) and by the intracellular injection of Ca^{2+} (Yada et al., 1986). The first purpose of the present study is to test this possibility by examining the effects of inhibitor of Ca^{2+}-activated K^+ channels in this cell line and by measuring the cytosolic free Ca^{2+} concentration during the hyperpolarizing responses with Ca^{2+}-selective microelectrodes.

VIP also induces a similar hyperpolarizing response via independent receptors in Intestine 407

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cells (Yada & Okada, 1984). VIP is known to act as an intestinal secretagogue by increasing cellular cyclic AMP (Schwartz et al., 1974; Klaeveman et al., 1975; Gaginella et al., 1978; Laburthe et al., 1979*a*,*b*; Binder, Lemp & Gardner, 1980; Dharmsathaphorn et al., 1983). Thus, it is possible that VIP-induced hyperpolarizing responses are mediated by cyclic AMP-activated K^+ channels (McRoberts, Beuerlein & Dharmsathaphorn, 1985; Ueda, Loo & Sachs, 1987). Alternatively, the hyperpolarizing response to VIP may be induced by Ca^{2+}-activated K^+ channels as a result of the intracellular Ca^{2+} release by increased cyclic AMP as found in chicken enterocytes (Semrad & Chang, 1987). The second purpose of the present study is to address these possibilities by investigating whether VIP induces a sizable increase in the cytosolic free Ca^{2+} and whether secretin and ATP, which also elevate cyclic AMP (Laburthe et al., 1979*a*,*b*; Binder et al., 1980; Korman et al., 1982), can induce a hyperpolarizing response in a similar manner in Intestine 407 cells.

Materials and Methods

The techniques for cell culture, cell fusion and intracellular recordings were identical with those described in a previous paper (Yada & Okada, 1984). The monolayer of giant Intestine 407 cells containing several tens of nuclei was produced by cell fusion with polyethyleneglycol. This helped to minimize the leakage artifact upon impalements with microelectrodes. The membrane potential was measured with 3 M KCl-filled microelectrodes (resistance, 20–100 MΩ; tip potential, ≤5 mV) at 35°C. The input membrane resistance was estimated by passing constant current pulses of 0.3 nA through the recording microelectrodes using a bridge circuit (WPI KS 700). Intestinal secretagogues were added directly to the bath solution or administered focally to the cell surface by pressure through a blunted micropipette which was located about 200 μm from the cell. To prevent osmotic perturbation which could activate volume-regulatory ion channels in the cells (Hazama & Okada, 1988), the secretagogues were dissolved in an isotonic saline solution prior to their applications. Reversal potentials of the secretagogue-induced responses were determined by applying currents through an additional microelectrode impaled simultaneously.

The cytosolic free Ca^{2+} concentration ($[Ca]_i$) was measured with a single-barreled Ca^{2+}-selective microelectrode connected to a differential high-input impedance amplifier (WPI FD 223) at 25°C. Ca^{2+}-selective microelectrodes were made with a neutral ligand sensor cocktail, as described previously (Yada et al., 1986; Oiki & Okada, 1988). The 90% response time was about 1 sec. The electrodes were calibrated before and after each impalement in Ca-EGTA buffer solutions (for the composition, *see* Oiki & Okada, 1988) at 25°C. Ca^{2+} concentrations in the buffer solutions were calculated by taking the purity of EGTA and the activity of H^+ into account. The Ca^{2+}-selective microelectrodes exhibiting Nernstian responses between pCa 7 and 3 were selected. The $[Ca]_i$ value was computed from the potential difference between the conventional and Ca^{2+}-selective microelectrodes.

The control bathing solution was a Tris-buffered saline solution containing (in mM): 143 NaCl, 4.2 KCl, 0.9 $CaCl_2$, 0.5 $MgCl_2$, 20 mannitol and 10 Tris-HCl (pH 7.3). A nominally Ca^{2+}-free solution was prepared by removing $CaCl_2$ from the control solution and was found to contain about 4 μM free Ca^{2+} when examined with Ca^{2+}-selective electrodes. The intestinal secretagogues examined in the present study were acetylcholine chloride (ACh: Nakarai Chemical), 5-hydroxytryptamine hydrochloride (5-HT: Sigma), histamine (Nakarai), synthetic porcine vasoactive intestinal peptide (VIP: Peninsula Laboratories or Protein Research Foundation), synthetic or native secretin (Protein Research Foundation or Boots) and ATP (Kohjin Chemical). Other drugs employed were adenosine (Kohjin), AMP (Boehringer Mannheim GmbH), ADP (Kohjin), dibutyryl cyclic AMP (db-cAMP, Sigma), adenylyl imidodiphosphate (AMP-PNP: Sigma), α,β-methylene ATP (Sigma), caffeine (Nakarai), KCN (Nakarai), *p*-trifluoro-methoxyphenylhydrazone (FCCP: Pierce Chemical), tetraethylammonium chloride (TEA: Nakarai), nonyltriethylammonium (C9: a gift from Dr. M. Tazawa, Tokyo University), quinine hydrochloride (Nakarai) and quinidine sulfate (Merck).

All data shown in the text are expressed as the mean ± SEM (*n*: number of cells observed). Differences between means were evaluated by the *F*-test.

Results

Hyperpolarizing Responses to Secretin and ATP via Separate Receptors

Intestine 407 cells responded to ACh, 5-HT, histamine and VIP with transient hyperpolarizations, as found previously (Yada & Okada, 1984). ATP and secretin also elicited similar hyperpolarizing responses at doses over 0.1 mM and 1 μM, respectively. Maximum responses were achieved with 1 mM ATP and 100 μM synthetic porcine secretin (or 20 units/liter Boots secretin) (Fig. 1). Their mean peak responses were −88.9 ± 2.8 (11) and −82.6 ± 1.4 mV (12), respectively. The hyperpolarizing responses were always associated with decreases in the membrane resistance (from the resting value of about 30 to about 10 MΩ at peak responses). The cells exhibited tachyphylaxis to the action of ATP or secretin after repeated administrations (Fig. 1*A*,*B*), as was the case for VIP or 5-HT (Fig. 1*C*,*D*) and for ACh or histamine (Yada & Okada, 1984). Desensitization produced by a given secretagogue did not impair the hyperpolarizing response to other secretagogues (Fig. 1*A–D*). Secretin and ATP were still effective when muscarinic receptors for ACh and H_1-receptors for histamine were antagonized by atropine and pyrilamine (Fig. 1*E*). Thus, the receptors for secretin and ATP appear to be independent of those for ACh, histamine, 5-HT and VIP.

The extracellular application of ADP also induced a similar hyperpolarizing response, whereas AMP or adenosine was ineffective (3–4 observa-

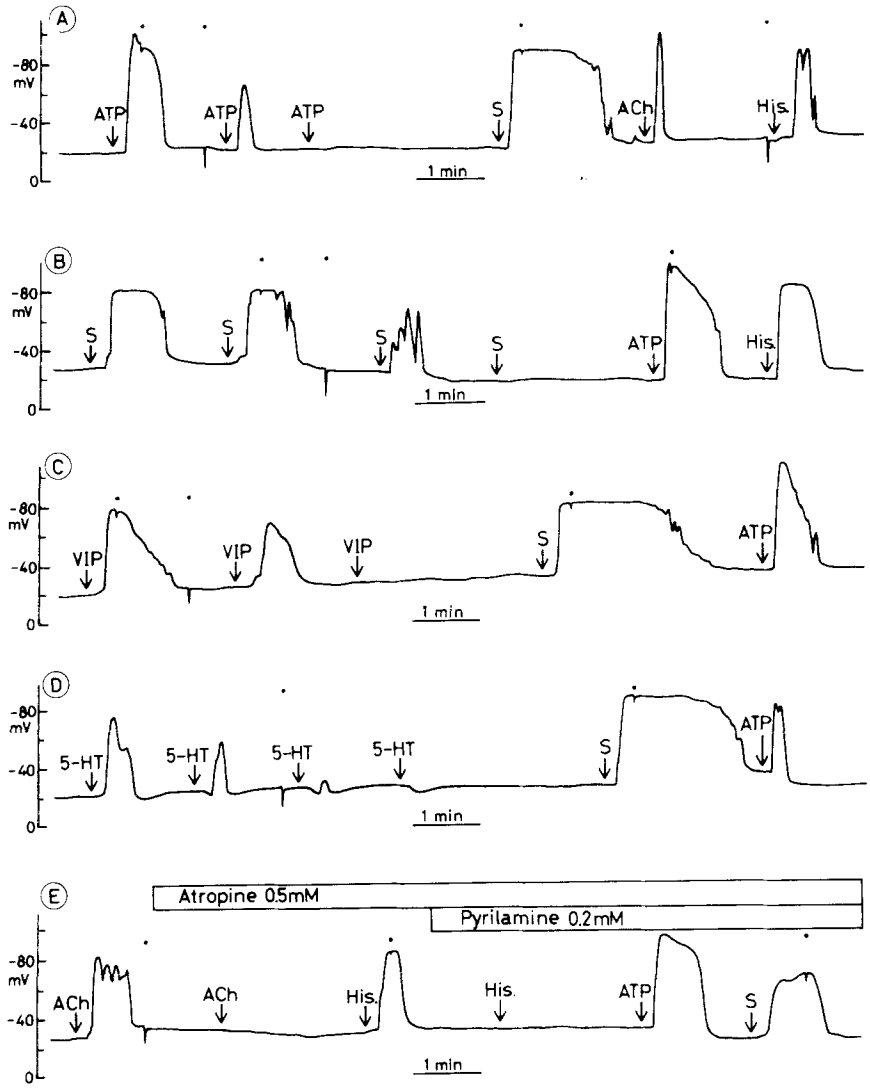

Fig. 1. Hyperpolarizing responses induced by ATP (1 mM) and secretin (*S*: 20 units/liter) as well as by acetylcholine (*ACh*: 1 mM), histamine (*His.*: 0.1 mM), serotonin (*5-HT*: 0.8 mM) and VIP (100 nM) in giant Intestine 407 cells. Arrows and rectangular bars indicate the application of agonists and drugs, respectively. Dots indicate the application of outward currents (0.3 nA) to monitor the input resistance. Note that decrement (desensitization) of the responses induced by repeated applications of a given agonist did not affect the responses to other agonists (*A–D*). Also, atropine or pyrilamine inhibited the responses to ACh or histamine, but not those to ATP and secretin (*E*). The latency of the response depended on the distance between the site of secretagogue application and the impaled cell, and hence reflected largely diffusion time of the agonists. The mean peak values of hyperpolarizing responses to secretin and ATP are given in the text, and those to other secretagogues were in a previous report (Table 1 in Yada & Okada, 1984). The mean duration of each secretagogue-induced hyperpolarizing response was as follows (in sec): 43 ± 7 (6) for ATP, 79 ± 10 (7) for secretin, 53 ± 15 (6) for ACh, 76 ± 30 (6) for histamine, 23 ± 8 (7) for 5-HT, and 56 ± 16 (7) for VIP

tions at 1 mM, *data not shown*). However, db-cAMP induced a transient hyperpolarization (the peak value: −64.0 ± 1.4 mV (5) at 1 mM). Nonhydrolysable ATP analogues, AMP-PNP and α,β-methylene ATP, were also effective in inducing similar responses (−80.8 ± 3.4 mV (8) and −84.6 ± 2.0 mV (7), respectively, at 1 mM).

Activation of Quinine-Sensitive K Conductance by Intestinal Secretagogues

A previous study showed that the hyperpolarizing responses to ACh, 5-HT, histamine and VIP are due to activation of the K^+ conductance (Yada & Okada, 1984). Since the input membrane resistance decreased during the hyperpolarizing responses to ATP and secretin (Fig. 1), similar ionic mechanisms may underlie these responses. To verify this inference, the reversal potentials for these responses were determined. As shown in Fig. 2, the reversal potential was estimated to be around −85 mV, which is close to the equilibrium potential for K^+ (−89 mV; Yada & Okada, 1984). This result indicates that an increase in K^+ conductance is the ionic basis for hyperpolarizing responses to ATP and secretin, as is the case for those to other secretagogues (Yada & Okada, 1984).

A K^+ channel blocker, quinine or quinidine (at doses over 0.1 mM), suppressed the hyperpolarizing response to every secretagogue examined. At 0.5–1 mM, the drug rapidly rendered the membrane insensitive to all the secretagogues (Fig. 3*A*,*B*), and the action was readily reversible (Fig. 3*B*). At a high dose (143 mM), another K^+ channel inhibitor, TEA, partially suppressed the hyperpolarizing responses (Fig. 3*C*), but the recovery upon washout of the drug was incomplete. C_9, a hydrophobic TEA ana-

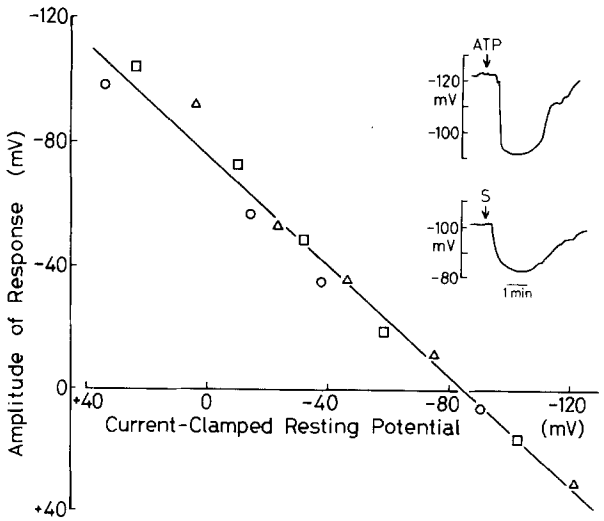

Fig. 2. Reversal potentials of the responses to ATP (1 mM: circles and triangles) and secretin (20 units/liter: squares) measured in three different giant cells. The amplitude of the response is plotted as a function of the membrane potential. *Inset:* Representative records of the responses to ATP (upper trace) and secretin (*S*: lower trace) at membrane potentials of −122 and −102 mV, respectively. Arrows are the same as in Fig. 1

logue (Armstrong, 1971), could abolish the ACh-induced hyperpolarizing response at 4 mM (two observations, *data not shown*). Quinine (or quinidine) is known to inhibit the Ca^{2+}-activated K^+ conductance in this cell line (Yada et al., 1986; Hazama & Okada, 1988), though not the case in an insulin-secreting cell line (Findlay et al., 1985). Therefore, it is probable that the Ca^{2+}-activated K^+ channels operate during the hyperpolarizing responses to intestinal secretagogues in Intestine 407 cells.

Increases in Cytosolic Free Ca by Individual Intestinal Secretagogues

If the secretagogue-induced hyperpolarizing responses are induced by Ca^{2+}-activated K^+ channels, an increase in the cytosolic free Ca^{2+} concentration ($[Ca]_i$) should occur concomitantly with the hyperpolarizing response. To test this inference, the $[Ca]_i$ and the membrane potential were simultaneously measured with a Ca^{2+}-selective microelectrode in combination with a conventional microelectrode in single giant Intestine 407 cells. The basal $[Ca]_i$ was always submicromolar. The mean value (0.69 ± 0.04 μM (29)) was greater than the value estimated by the quin2 method in nonfused Intestine 407 cells (Sjölander, 1988) or in isolated chicken enterocytes (Semrad & Chang, 1987; Chang et al., 1986), but similar to those measured in a variety of epithelial cells by means of Ca^{2+}-selective microelectrodes (Lee, Taylor & Windhager, 1980; O'Doherty, Youmans & Armstrong, 1980; O'Doherty & Stark, 1981; O'Doherty et al., 1983; Kelepouris, Agus & Civan, 1985). As shown in Fig. 4, when the intestinal secretagogues, which are believed to act via increased Ca^{2+}, were applied the $[Ca]_i$ level transiently increased from the submicromolar basal level to the micromolar concentrations. The mean peak values of the responses to ACh (1 mM), 5-HT (2 mM) and histamine (0.1 mM) were 1.67 ± 0.21 (5), 1.13 ± 0.22 (3), and 1.40 ± 0.10 μM (7), respectively. Similar Ca^{2+} responses were also induced by stimulation with the secretagogues which are known to increase cyclic AMP (Fig. 5). The peak responses were 1.55 ± 0.19 (3), 1.54 ± 0.68 (3) and 1.58 ± 0.17 μM (8) for stimulations with VIP (0.5 μM), secretin (100 μM) and ATP (1 mM), respectively. The differences between the mean basal $[Ca]_i$ and the mean peak $[Ca]_i$ value upon stimulation with each secretagogue were significant at $P < 0.005$, whereas the mean peak values of secretagogue-induced $[Ca^{2+}]_i$ responses were not statistically different from each other ($P > 0.05$).

The secretagogue-induced $[Ca]_i$ increase was always in phase with the membrane hyperpolarization (Figs. 4 & 5). The Ca^{2+} response did not result from the membrane hyperpolarization or from the poor voltage sensitivity of Ca^{2+}-selective microelectrodes, because hyperpolarization produced by a current pulse injection did not influence the steady level of $[Ca]_i$ (Fig. 5*A*), and because quinine did not affect the $[Ca]_i$ increase in response to ATP in spite of elimination of the hyperpolarizing response by the drug (2 observations, *data not shown*).

Origin of Increased Cytosolic Ca by Intestinal Secretagogues

When the extracellular Ca^{2+} concentration was reduced to around pCa 7 by adding 10 μM EGTA to a nominally Ca^{2+}-free solution, the hyperpolarizing responses to ACh and 5-HT were preferentially inhibited (Fig. 6*A*). The EGTA effects were reversible, though it took more than 30 min to wash out. The application of Co^{2+} (20–60 mM), an inorganic antagonist of Ca^{2+} channels (Hagiwara & Takahashi, 1967; Baker, Meves & Ridgway, 1973), to the standard bathing solution also abolished only the ACh- and 5-HT-induced responses (Fig. 6*B*). The responses to both secretagogues were restored rapidly after removal of Co^{2+}. In contrast to the hyperpolarizing responses to ACh and 5-HT, those to histamine, VIP, secretin and ATP persisted even in the presence of EGTA or Co^{2+} (Fig. 6*A*,*B*). Thus, it appears that the source of Ca^{2+} mobilized to the cytoplasm upon stimulation with ACh or 5-HT was extracellular, whereas that for the responses to other secretagogues was intracellular.

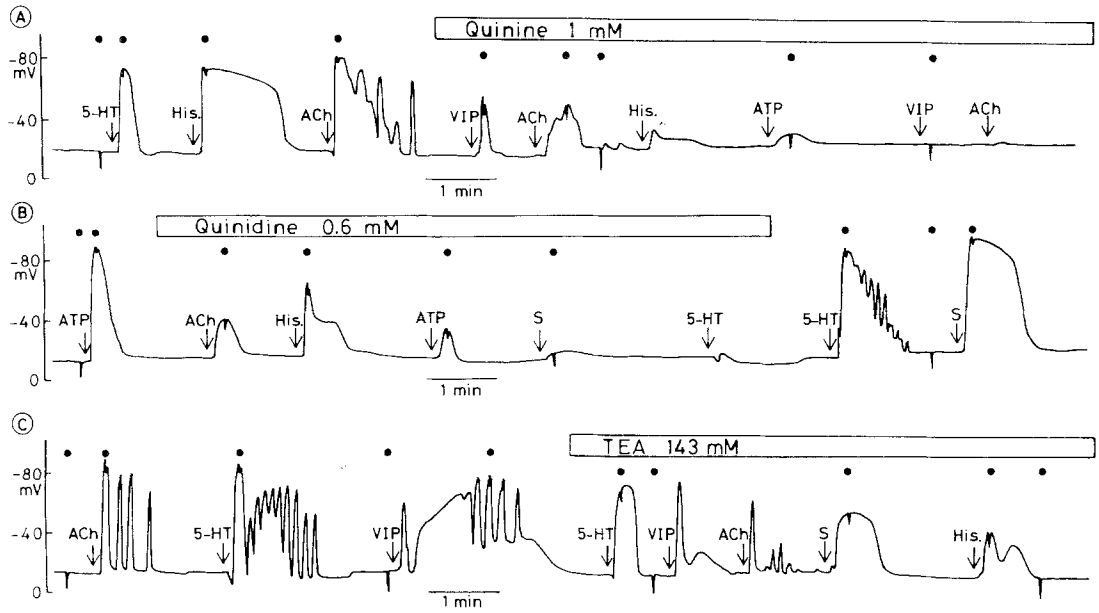

Fig. 3. Effects of K^+ channel blockers, 1 mM quinine (*A*), 0.6 mM quinidine (*B*) and 143 mM TEA (*C*), on the secretagogue-induced hyperpolarizing responses in giant Intestine 407 cells. Arrows, rectangular bars and dots are the same as in Fig. 1. The doses and abbreviations of secretagogues employed are the same as in Fig. 1. Each trace represents three to eight similar experiments

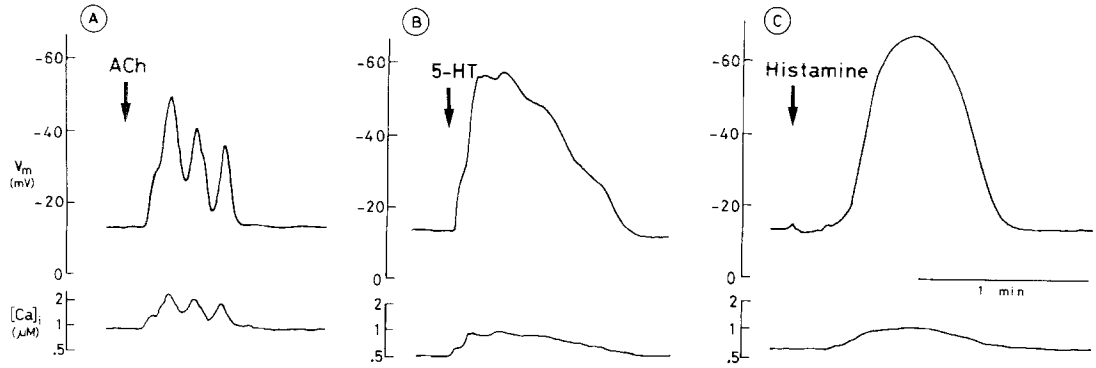

Fig. 4. Changes in the cytosolic free Ca^{2+} concentration ($[Ca]_i$: lower traces) in giant Intestine 407 cells during hyperpolarizing responses (upper traces) to 1 mM ACh (*A*), 0.1 mM histamine (*B*) and 2 mM 5-HT (*C*). Arrows are the same as in Fig. 1. Data represent three to seven similar experiments

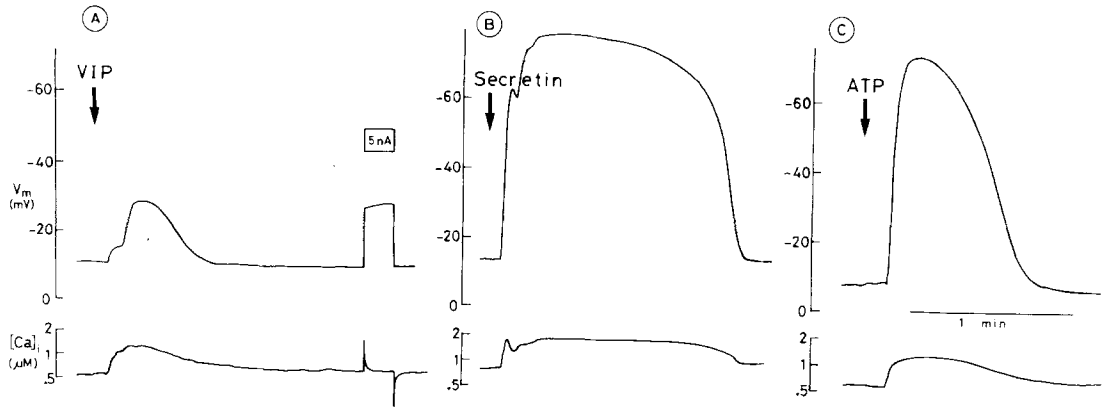

Fig. 5. $[Ca]_i$ increases (lower traces) during hyperpolarizing responses (upper traces) to 0.5 μM VIP (*A*), 100 μM synthetic secretin (*B*) and 1 mM ATP (*C*). Arrows are the same as in Fig. 1. In *A* a 5 nA current pulse (rectangular bar) was applied to the cell through an intracellular electrode after compensating for the electrode resistance with a bridge circuit. Note that the response time to potential changes was satisfactorily fast and that the measured steady $[Ca]_i$ level was not affected by the current-induced hyperpolarization. Data represent three to eight similar experiments

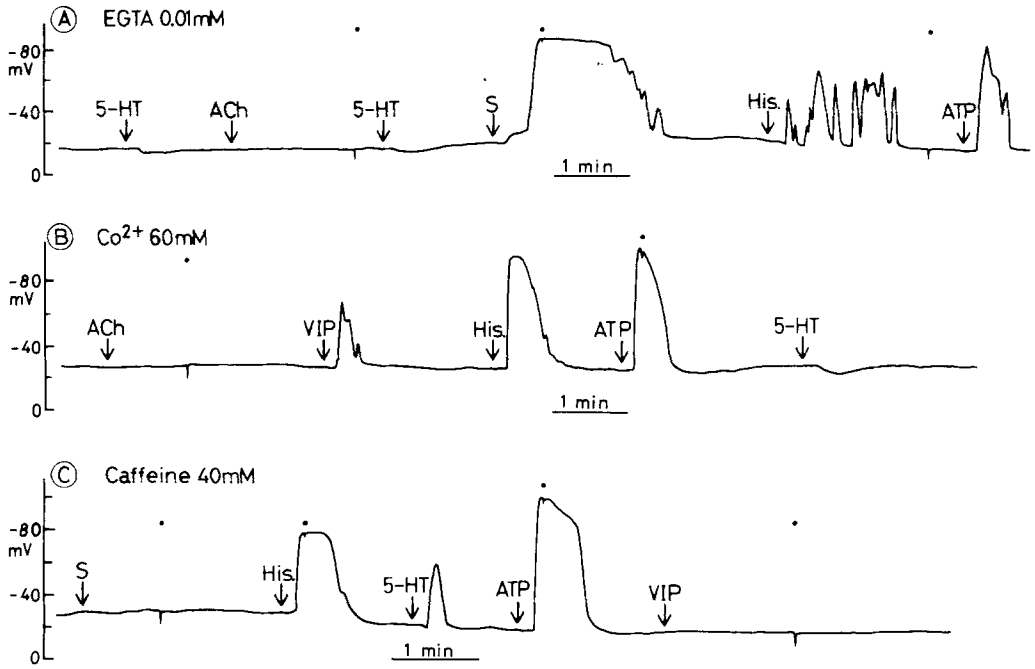

Fig. 6. Effects of extracellular EGTA (*A*), Co^{2+} (*B*) and caffeine (*C*) on the hyperpolarizing responses to secretagogues. EGTA (10 μM) was added to a nominally Ca^{2+}-free bathing solution about 10 min before the impalement. Co^{2+} (60 mM) and caffeine (40 mM) were added to the control bathing solution (containing 0.9 mM Ca^{2+}) about 1 and 10 min before the impalement, respectively. Arrows, dots, the doses and abbreviations of secretagogues are the same as in Fig. 1. Note that the 5-HT and ACh responses were abolished by EGTA and Co^{2+}, whereas the secretin and VIP responses were blocked by caffeine. Data represent three to five similar experiments

Intracellular Ca^{2+} pools consist of mitochondrial and nonmitochondrial structures in enterocytes (Velasco et al., 1986; Ilundain et al., 1987; Sepulveda & Smith, 1987; van Corven et al., 1987). Mitochondrial inhibitors, such as KCN (2 mM), NaN_3 (5 mM) and FCCP (2 μM), were without effect (up to 60 min) on the secretagogue-induced hyperpolarizing responses (3 to 7 observations for each secretagogue, *data not shown*). In contrast, caffeine, which is known to facilitate the Ca^{2+} release from sarcoplasmic or endoplasmic reticulum (Weber, 1968; Endo, 1975; Moore & Pastan, 1977), selectively inhibited the hyperpolarizing responses to VIP and secretin (Fig. 6*C*). These results suggest that caffeine-sensitive nonmitochondrial Ca^{2+} stores are responsible for the Ca^{2+} release upon stimulation with VIP or secretin.

Discussion

Taken together with our previous work (Yada & Okada, 1984), the present data indicate that a human intestinal epithelial cell line, Intestine 407, retains separate receptors capable of responding to ATP, secretin, VIP, ACh, 5-HT and histamine, which have all been identified as neurohumoral factors stimulating small intestinal secretion (Cooke, 1987; Donowitz & Welsh, 1987). Two classes of membrane receptors may generally be distinguished: those coupled to phospholipase C, activation of which generates inositol trisphosphate (IP_3) and diacylglycerol, thereby triggering the regulatory process mediated by Ca^{2+} and protein kinase C (Nishizuka, 1984), and those to adenylate cyclase catalyzing the cyclic AMP production. In epithelial cells of the small intestine, the receptors of ACh, 5-HT and histamine may belong to the former class, since the action of these agonists are known to be mediated by cytosolic Ca^{2+} ions (Bolton & Field, 1977; Hardcastle, Hardcastle & Redfern, 1981; Chang et al., 1986; Donowitz & Welsh, 1987; Hardcastle & Hardcastle, 1987). On the other hand, VIP, secretin and ATP may be the agonists for the latter class, since an increase in the cellular cyclic AMP or activation of adenylate cyclase was observed in enterocytes upon stimulation with these secretagogues (Schwartz et al., 1974; Klaeveman et al., 1975; Laburthe et al., 1979*a*,*b*; Binder et al., 1980; Korman et al., 1982; Dharmsathaphorn et al., 1983). Secretin shares a structural homology with VIP and actually binds to the VIP receptor with a low affinity (Laburthe et al., 1979*b*). In the present study, in fact, much higher doses (1 to 100 μM) of secretin were needed to induce hyperpolarizing responses in Intestine 407 cells than those of VIP (1 to 100 nM). Thus, one could argue that the secretin-induced response might be induced by activation of the VIP

receptor rather than its own receptor. However, this possibility was ruled out by the fact that secretin did induced the response even after desensitization of the response to VIP (Fig. 1*C*).

As expected, the present study with Ca^{2+}-selective and conventional microelectrodes showed that ACh, 5-HT and histamine induced significant increases in the cytosolic free Ca^{2+} concentration and hyperpolarizations, in phase, due to activation of the Ca^{2+}-dependent K^+ conductance in Intestine 407 cells (Fig. 4). The Ca^{2+} ions mobilized upon stimulation with ACh or 5-HT appear to be derived mainly from the extracellular space, presumably via Ca^{2+} channels, because the hyperpolarizing responses to both agonists were selectively abolished by a reduction in the extracellular Ca^{2+} concentration down to around pCa 7 or by application of a Ca^{2+} channel blocker, Co^{2+} (Fig. 6*A*,*B*). If phospholipase C activates were associated with these receptors, activation of protein kinase C due to the production of diacylglycerol would participate in the opening of Ca^{2+} channels, as noted in a number of cell species (DeRiemer et al., 1985; Osugi et al., 1986; Strong et al., 1987; Yamaguchi, Kleeman & Muallem, 1987; Yada, Russo & Sharp, 1989). Alternatively, IP_3, another product of the activated phophoslipase C, may induce the opening of Ca^{2+}-permeable channels, as found in several cell species (Kuno & Gardner, 1987; Parker & Miledi, 1987; Penner, Matthews & Neher, 1988; Vilven & Coronado, 1988). On the other hand, the experiments with EGTA and Co^{2+} (Figs. 6*A*,*B*) strongly suggest that the origin of Ca^{2+} ions mobilized upon stimulation with histamine is intracellular. This is in contrast to the observation that histamine-induced Cl^- secretion is inhibited by deprivation of serosal Ca^{2+} ions or by the application of varapamil in rat small intestine (Hardcastle & Hardcastle, 1987), but in good agreement with the finding that histamine-induced Ca^{2+} mobilization is not affected by deprivation of the extracellular Ca^{2+} ions in colonic T_{84} cells (Wasserman et al., 1988). Since IP_3 is known to provoke the Ca^{2+} release from intracellular nonmitochondrial stores in permeabilized enterocytes (Velasco et al., 1986; Ilundain et al., 1987; van Corven et al., 1987), histamine-induced responses might be mediated by IP_3.

Contrary to what we had predicted, VIP, secretin and ATP, which are all known to elevate cellular cyclic AMP, were found to induce increases in the cytosolic free Ca^{2+} concentration in Intestine 407 cells (Fig. 5). There is a possibility that receptors for these secretagogues are coupled not only to stimulate adenylate cyclase activity but also to stimulate inositol phospholipid breakdown, as shown in the secretin receptor of pancreatic acini (Trimble et al., 1986) and in the glucagon receptor of hepatocytes (Wakelam et al., 1986). Alternatively, the increased cyclic AMP level may somehow induce an increase in the $[Ca]_i$ within enterocytes, as suggested in previous reports (Frizzell, 1977; Donowitz, 1983), since db-cAMP was found to induce a hyperpolarization in Intestine 407. Recently, sizable $[Ca]_i$ increases were, in fact, observed upon the application of 8-bromo-cyclic AMP in isolated chicken enterocytes by means of quin2 (Semrad & Chang, 1987). Furthermore, our preliminary experiments with Ca^{2+}-selective microelectrodes showed that dibutyryl cyclic AMP (1 mM) increases in cytosolic free Ca^{2+} in giant Intestine 407 cells (Y. Okada, S. Ueda, S. Oiki and T. Yada, *unpublished observations*). Although cyclic AMP has been suggested to release Ca^{2+} from mitochondria (Juzu & Holdsworth, 1980), mitochondrial inhibitors failed to impair the hyperpolarizing responses to cyclic AMP-mediated secretagogues in Intestine 407 cells. Ca^{2+} ions mobilized upon stimulation with VIP and secretin appear to originate from the microsomal store since the prior treatment with caffeine preferentially abolished VIP- and secretin-induced hyperpolarizing responses (Fig. 6*C*).

The secretagogue-induced hyperpolarizing responses recorded from fused giant cells in the present study were also observed in nonfused small Intestine 407 cells by conventional intracellular recordings (Yada & Okada, 1984) and by whole-cell recordings with patch electrodes (A. Itoh and Y. Okada, *unpublished observations*). The hyperpolarizing responses were due to increases in the K^+ conductance (Fig. 2) and were sensitive to quinine or quinidine (Fig. 3), blockers of Ca^{2+}-activated K^+ channels in this cell line (Yada et al., 1986; Hazama & Okada, 1988). The hyperpolarizing responses were consistently associated with significant increases in $[Ca]_i$ (Figs. 4 and 5). In addition, the hyperpolarizing responses to ACh, 5-HT, VIP and secretin were inhibited when secretagogue-induced Ca^{2+} mobilization was suppressed (with EGTA for ACh or 5-HT, and with caffeine for VIP or secretin; Fig. 6). Therefore, Ca^{2+}-activated K^+ channels seem to be the common target activated by intestinal secretagogues, regardless of whether their second messengers are Ca^{2+} or cyclic AMP. The presence of Ca^{2+}-activated K^+ channels has been demonstrated by the patch-clamp studies in this cell line (Hazama & Okada, 1988) and in isolated enterocytes (Morris, Gallacher & Lee, 1986; Sheppard, Giraldez & Sepúlveda, 1988). This agrees with the observation that ACh and 5-HT activated the basolateral Ca^{2+}-dependent K^+ conductance in rat mid-intestine (Hardcastle & Hardcastle, 1986). Similar Ca^{2+}-mediated activation of the basolateral K^+ conductance was reported for other Cl^--secreting epithelia, such as colonic T_{84} cells (Cartwright et al., 1985; Dharmsathaphorn & Pandol, 1986; Mandel et al., 1986; Wasserman et al., 1988) and canine

trachea (Welsh, Smith & Frizzell, 1982; 1983). It is well known that an increase in the K^+ conductance is prerequisite to epithelial secretory functions (Petersen, 1986; Donowitz & Welsh, 1987). Presumably, this process may provide an electrical driving force favorable for electroconductive Cl^- exit across the luminal membrane and/or K^+ recycling which, in turn, creates a favorable condition for Cl^- entry via Na^+-K^+-Cl_2^- cotransporters across the basolateral membrane.

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References

Armstrong, C.M. 1971. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. Gen. Physiol.* **55:**413–437

Baker, P.F., Meves, H., Ridgway, E.B. 1973. Effects of manganese and other agents on the calcium uptake that follows depolarization of squid axons. *J. Physiol. (London)* **231:**511–526

Binder, H.J., Lemp, G.F., Gardner, J.D. 1980. Receptors for vasoactive intestinal peptide and secretin on small intestinal epithelial cells. *Am. J. Physiol.* **238:**G190–G196

Bolton, J.E., Field, M. 1977. Ca ionophore-stimulated ion secretion in rabbit ileal mucosa: Relation to actions of cyclic 3′,5′-AMP and carbamylcholine. *J. Membrane Biol.* **35:**159–174

Cartwright, C.A., McRoberts, J.A., Mandel, K.G., Dharmsathaphorn, K. 1985. Synergistic action of cyclic adenosine monophosphate- and calcium-mediated chloride secretion in a colonic epithelial cell line. *J. Clin. Invest.* **76:**1837–1842

Chang, E.B., Brown, D.R., Wang, N.S., Field, M. 1986. Secretagogue-induced changes in membrane calcium permeability in chicken and chinchilla ileal mucosa. *J. Clin. Invest.* **78:**281–287

Cooke, H.J. 1987. Neural and humoral regulation of small intestinal electrolyte transport. *In:* Physiology of Gastrointestinal Tract. L.R. Johnson, editor. pp. 1307–1350. Raven, New York

DeRiemer, S.A., Strong, J.A., Albert, K.A., Greengard, P., Kaczmarek, L.K. 1985. Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. *Nature (London)* **313:**313–316

Dharmsathaphorn, K., Harms, V., Yamashiro, D.H., Hughes, R.J., Binder, H.J., Wright, E.M. 1983. Preferential binding of vasoactive intestinal polypeptide to basolateral membrane of rat and rabbit enterocytes. *J. Clin. Invest.* **71:**27–35

Dharmsathaphorn, K., Pandol, S.J. 1986. Mechanism of chloride secretion induced by carbachol in a colonic epithelial cell line. *J. Clin. Invest.* **77:**348–354

Donowitz, M. 1983. Ca^{2+} in the control of active Na and Cl transport: Involvement in neurohumoral action. *Am. J. Physiol.* **245:**G165–G177

Donowitz, M., Welsh, M.J. 1987. Regulation of mammalian small intestinal electrolyte secretion. *In:* Physiology of Gastrointestinal Tract. L.R. Johnson, editor. pp. 1351–1388. Raven, New York

Endo, M. 1975. Mechanisms of action of caffeine on the sarcoplasmic reticulum of skeletal muscle. *Proc. Jpn. Acad.* **51:**479–484

Findlay, I., Dunne, M.J., Ullrich, S., Wollheim, C.B., Petersen, O.H., 1985. Quinine inhibits Ca^{2+}-independent K^+ channels whereas tetraethylammonium inhibits Ca^{2+}-activated K^+ channels in insulin-secreting cells. *FEBS Lett.* **185:**4–8

Frizzell, R.A. 1977. Active chloride secretion by rabbit colon: Calcium dependent stimulation by ionophore A23187. *J. Membrane Biol.* **35:**175–187

Gaginella, T.S., Phillips, S.F., Dozois, R.R., Go, V.L.W. 1978. Stimulation of adenylate cyclase in homogenates of isolated intestinal epithelial cells from hamsters. Effects of gastrointestinal hormones, prostaglandins, and deoxycholic and ricinoleic acids. *Gastroenterology* **74:**11–15

Hagiwara, S., Takahashi, K. 1967. Surface density of calcium ions and calcium spikes on the barnacle muscle fiber membrane. *J. Gen. Physiol.* **50:**583–601

Hardcastle, J., Hardcastle, P.T. 1986. The involvement of basolateral potassium channels in the intestinal response to secretagogues in the rat. *J. Physiol. (London)* **379:**331–345

Hardcastle, J., Hardcastle, P.T. 1987. The secretory actions of histamine in rat small intestine. *J. Physiol. (London)* **388:**521–532

Hardcastle, J., Hardcastle, P.T., Redfern, J.S. 1981. Action of 5-hydroxytryptamine on intestinal ion transport in the rat. *J. Physiol. (London)* **320:**41–55

Hazama, A., Okada, Y. 1988. Ca^{2+} sensitivity of volume-regulatory K^+ and Cl^- channels in cultured human epithelial cells. *J. Physiol. (London)* **402:**687–702

Hazama, A., Yada, T., Okada, Y. 1985. HeLa cells have histamine H1-receptors which mediate activation of the K^+ conductance. *Biochim. Biophys. Acta* **845:**249–253

Henle, G., Deinhardt, F. 1957. The establishment of strains of human cells in tissue culture. *J. Immunol.* **79:**54–59

Hughes, J.M., Murad, F., Chang, B., Guerrant, R.L. 1978. Role of cyclic GMP in the action of heat-stable enterotoxin of *Escherichia coli*. *Nature (London)* **271:**755–756

Ilundain, A., O'Brien, J.A., Burton, K.A., Sepulveda, F.V. 1987. Inositol trisphosphate and calcium mobilisation in permeabilised enterocytes. *Biochim. Biophys. Acta* **896:**113–116

Itoh, A., Ueda, S., Okada, Y. 1989. Chloride current activation induced by intestinal secretagogues in an intestinal epithelial cell line. *Jpn. J. Physiol. (Abstr.) (in press)*

Juzu, H.A., Holdsworth, E.S. 1980. New evidence for the role of cyclic AMP in the release of mitochondrial calcium. *J. Membrane Biol.* **52:**185–186

Kelepouris, E., Agus, Z.S., Civan, M.M. 1985. Intracellular calcium activity in split frog skin epithelium: Effect of cAMP. *J. Membrane Biol.* **88:**113–121

Klaeveman, H.L., Conlon, T.P., Levy, A.G., Garoner, J.D. 1975. Effects of gastrointestinal hormones on adenylate cyclase activity in human jejunal mucosa. *Gastroenterology* **68:**667–675

Korman, L.Y., Lemp, G.F., Jackson, M.J., Gardner, J.D. 1982. Mechanism of action of ATP on intestinal epithelial cells. Cyclic AMP-mediated stimulation of active ion transport. *Biochim. Biophys. Acta* **721:**47–54

Kuno, M., Gardner, P. 1987. Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature (London)* **326:**301–304

Laburthe, M., Mangeat, P., Marchis-Mouren, G., Rosselin, G. 1979*a*. Activation of cyclic AMP-dependent protein kinases by vasoactive intestinal peptide (VIP) in isolated intestinal epithelial cells from rat. *Life Sci.* **25:**1931–1938

Laburthe, M., Prieto, J.C., Amiranoff, B., Dupont, C., Hui Bon Hoa, D., Rosselin, G. 1979*b*. Interaction of vasoactive intestinal peptide with isolated intestinal epithelial cells from rat. 2. Characterization and structural requirements of the stimulatory effect of vasoactive intestinal peptide on production of 3′:5′-monophosphate. *Eur. J. Biochem.* **96:**239–248

Lee, C.O., Taylor, A., Windhager, E.E. 1980. Cytosolic calcium ion activity in epithelial cells of *Necturus* kidney. *Nature (London)* **287:**859–861

Mandel, K.G., McRoberts, J.A., Beuerlein, G., Foster, E.S., Dharmsathaphorn, K., 1986. Ba^{2+} inhibition of VIP- and A23187-stimulated Cl^- secretion by T_{84} cell monolayer. *Am. J. Physiol.* **250:**C486–C494

McRoberts, J.A., Beuerlein, G., Dharmsathaphorn, K. 1985. Cyclic AMP and Ca^{2+}-activated K^+ transport in a human colonic epithelial cell line. *J. Biol. Chem.* **260:**14163–14172

Moore, L., Pastan, I. 1977. Energy-dependent calcium uptake activity in cultured mouse fibroblast microsomes. Regulation of the uptake system by cell density. *J. Biol. Chem.* **252:**6304–6309

Morris, A.P., Gallacher, D.V., Lee, J.A.C. 1986. A large conductance, voltage- and calcium-activated K^+ channel in the basolateral membrane of rat enterocytes. *FEBS Lett.* **206:**87–92

Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (London)* **308:**693–698

O'Doherty, J., Stark, R.J. 1981. Transmembrane and transepithelial movement of calcium during stimulus-secretion coupling. *Am. J. Physiol.* **241:**G150–G158

O'Doherty, J., Stark, R.J., Crane, S.J., Brugge, K.L. 1983. Changes in cytosolic calcium during cholinergic and adrenergic stimulation of the parotid salivary gland. *Pfluegers Arch.* **398:**241–246

O'Doherty, J., Youmans, S.J., Armstrong, W.McD. 1980. Calcium regulation during stimulus-secretion coupling: Continuous measurement of intracellular calcium activities. *Science* **209:**510–513

Oiki, S., Okada, Y. 1988. Clq induces chemotaxis and K^+ conductance activation coupled to increased cytosolic Ca^{2+} in mouse fibroblasts. *J. Immunol.* **141:**3177–3185

Okada, Y., Hazama, A., Yada, T. 1985. HeLa cells and Intestine 407 cell. Their differences in electrical membrane responses to secretagogues and in ecto-enzyme activities. *Cell Struct. Funct.* **10:**515p (*Abstr.*)

Osugi, T., Imaizumi, T., Mizushima, A., Uchida, S., Yoshida, H. 1986. 1-Oleoyl-2-acetyl-glycerol and phorbol diester stimulate Ca^{2+} influx through Ca^{2+} channels in neuroblastoma × glioma hybrid NG108-15 cells. *Eur. J. Pharmacol.* **126:**47–51

Parker, I., Miledi, R. 1987. Inositol trisphosphate activates a voltage-dependent calcium influx in *Xenopus* oocytes. *Proc. R. Soc. London B* **231:**27–36

Penner, R., Matthews, G., Neher, E. 1988. Regulation of calcium influx by second messengers in rat mast cells. *Nature (London)* **334:**499–504

Petersen, O.H. 1986. Potassium channels and fluid secretion. *News Physiol. Sci.* **1:**92–95

Schwartz, C.J., Kimberg, D.V., Sheerin, H.E., Field, M., Said, S.I. 1974. Vasoactive intestinal peptide stimulation of adenylate cyclase and active electrolyte secretion in intestinal mucosa. *J. Clin. Invest.* **54:**536–544

Semrad, C.E., Chang, E.G. 1987. Calcium-mediated cyclic AMP inhibition of Na-H exchange in small intestine. *Am. J. Physiol.* **252:**C315–C322

Sepulveda, F.V., Smith, S.M. 1987. Calcium transport by permeabilised rabbit small intestinal epithelial cells. *Pfluegers Arch.* **408:**231–238

Sheppard, D.N., Giraldez, F., Sepúlveda, F.V. 1988. Kinetics of voltage- and Ca^{2+} activation and Ba^{2+} blockade of a large-conductance K^+ channel from *Necturus* enterocytes. *J. Membrane Biol.* **105:**65–75

Sjölander, A. 1988. Direct effects of wheat germ agglutinin on inositol phosphate formation and cytosolic-free calcium level in Intestine 407 cells. *J. Cell. Physiol.* **134:**473–478

Strong, J.A., Fox, A.P., Tsien, R.W., Kaczmarek, L.K. 1987. Stimulation of protein kinase C recruits convert calcium channels in *Aplysia* bag cell neurons. *Nature (London)* **325:**714–717

Thomas, D.D., Knoop, F.C. 1983. Effect of heat-stable enterotoxin of *Escherichia coli* on cultured mammalian cells. *J. Infect. Dis.* **147:**450–459

Trimble, E.R., Burzzone, R., Biden, T.J., Farese, R.V. 1986. Secretin induces rapid increases in inositol triphosphate, cytosolic Ca^{2+} and diacylglycerol as well as cyclic AMP in rat pancreatic acini. *Biochem. J.* **239:**257–261

Ueda, S., Loo, D.D.F., Sachs, G. 1987. Regulation of K^+ channels in the basolateral membrane of *Necturus* oxyntic cells. *J. Membrane Biol.* **97:**31–41

van Corven, E.J.J.M., Verbost, P.M., de Jong, M.D., van Os, C.H. 1987. Kinetics of ATP-dependent Ca^{2+} uptake by permeabilized rat enterocytes. Effects of inositol 1,4,5-trisphosphate. *Cell Calcium* **8:**197–206

Velasco, G., Shears, S.B., Michell, R.H., Lazo, P.S. 1986. Calcium uptake by intracellular compartments in permeabilised enterocytes. Effect of inositol 1,4,5 trisphosphate. *Biochem. Biophys. Res. Commun.* **139:**612–618

Vilven, J., Coronado, R. 1988. Opening of dihydropyridine calcium channels in skeletal muscle membranes by inositol trisphosphate. *Nature (London)* **336:**587–589

Wakelam, M.J.O., Murphy, G.J., Hruby, V.J., Houslay, M.D. 1986. Activation of two signal-transduction systems in hepatocytes by glucagon. *Nature (London)* **323:**68–71

Wasserman, S.I., Barrett, K.E., Huott, P.A., Beuerlein, G., Kagnoff, M.F., Dharmsathaphorn, K. 1988. Immune-related intestinal Cl^- secretion I. Effect of histamine on the T_{84} cell line. *Am. J. Physiol.* **254:**C53–C62

Weber, A. 1968. The mechanism of the action of caffeine on sarcoplasmic reticulum. *J. Gen. Physiol.* **52:**760–772

Welsh, M.J., Smith, P.L., Frizzell, R.A. 1982. Chloride secretion by canine tracheal epithelium: II. The cellular electrical potential profile. *J. Membrane Biol.* **70:**227–238

Welsh, M.J., Smith, P.L., Frizzell, R.A. 1983. Chloride secretion by canine tracheal epithelium: III. Membrane resistances and electromotive forces. *J. Membrane Biol.* **71:**209–218

Yada, T., Oiki, S., Ueda, S., Okada, Y. 1986. Synchronous oscillation of the cytoplasmic Ca^{2+} concentration and membrane potential in cultured epithelial cells (Intestine 407). *Biochim. Biophys. Acta* **887:**105–112

Yada, T., Okada, Y. 1984. Electrical activity of an intestinal epithelial cell line: Hyperpolarizing responses to intestinal secretagogues. *J. Membrane Biol.* **77:**33–44

Yada, T., Russo, L.L., Sharp, G.W.G. 1989. Phorbol ester-stimulated insulin secretion by RINm5F insulinoma cells is linked with depolarization and an increase in cytosolic free Ca^{2+} concentration. *J. Biol. Chem.* **264:**2455–2462

Yamaguchi, D.T., Kleeman, C.R., Muallem, S. 1987. Protein kinase C-activated calcium channel in the osteoblast-like clonal osteosarcoma cell line UMR-106. *J. Biol. Chem.* **262:**14967–14973

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